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Characterizing the Role of 1p36 Deletion in Breast Cancer and Identifying Candidate Tumor Suppressors

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13. SUPPLEMENTARY NOTES

14. ABSTRACT Subject: Over 60% of human breast tumors display a deletion of one copy of the 1p36 region of the short arm of chromosome 1. Patients whose tumors carry this deletion show a three-fold increase in mortality, suggesting a biological role for this deletion in tumor development, and suggesting the presence of one or more tumor suppressors in this region. Purpose: Characterization of the unique biology of tumors with 1p36 deletion, and identification of the tumor suppressor(s) in the region may inform therapeutic strategies, and present unique therapeutic targets for this subset of breast cancer cases with relatively poor survival. Scope: The goals of this research project are to 1) develop a mouse model for 1p36 deletion in breast cancer by generating mice harboring loxP sequences flanking the deletion region, and crossing to tissue-specific Cre expressing mice, 2) perform in-vivo insertional mutagenesis in breast tumors using the two-component Sleeping Beauty transposon system (mutagenic transposons mobilized by a trans-acting transposase) to tag tumor suppressors and oncogenes during tumor development and 3) to combine these two systems to identify tumor suppressors in the 1p36 region. Technical setbacks and the observation by another group that deletion of at least part of the 1p36 region systemically does not lead to breast tumors in mice have led us to focus primarily on the Sleeping Beauty system for the last two years. We have opted for an in vitro approach in which we use lentiviral constructs to activate transposons in mammary epithelial cells, which we then transplant into recipient mice.

15. SUBJECT TERMS
1p36, insertional mutagenesis, sleeping beauty, mouse models, tumor suppressors

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FINAL STATUS REPORT: BC050930 Characterizing the Role of 1p36 Deletion in Breast Cancer and Identifying Candidate Tumor Suppressors

INTRODUCTION: **Subject:** Over 60% of human breast tumors display a deletion of one copy of the 1p36 region of the short arm of chromosome 1. Patients whose tumors carry this deletion show a three-fold increase in mortality, suggesting a biological role for this deletion in tumor development, and suggesting the presence of one or more tumor suppressors in this region. **Purpose:** Characterization of the unique biology of tumors with 1p36 deletion, and identification of the tumor suppressor(s) in the region may inform therapeutic strategies, and present unique therapeutic targets for this subset of breast cancer cases with relatively poor survival. **Scope:** The goals of this research project are to 1) develop a mouse model for 1p36 deletion in breast cancer by generating mice harboring loxP sequences flanking the deletion region, and crossing to tissue-specific Cre expressing mice, 2) perform in-vivo insertional mutagenesis in breast tumors using the two-component Sleeping Beauty transposon system (mutagenic transposons mobilized by a trans-acting transposase) to tag tumor suppressors and oncogenes during tumor development and 3) to combine these two systems to identify tumor suppressors in the 1p36 region. Technical setbacks and the observation by another group that deletion of at least part of the 1p36 region systemically does not lead to breast tumors in mice have led us to focus primarily on the Sleeping Beauty system for the last two years. While we have been able to map transposon insertions, we have established that existing mouse constructs were inadequate to drive or accelerate tumors in the mammary gland. We have therefore opted for an in vitro approach in which we use lentiviral constructs to activate transposons in mammary epithelial cells, which we then transplant into recipient mice.

BODY: Results from all Tasks from which we have outcomes to date from the approved Statement of Work are summarized below:

Task 1: Development of a mouse model for 1p36 deletion

Aims 1A, 1B, and 1C: Obtain and verify MICER loxP targeting clones flanking syntenic region of chromosome 4 corresponding to 1p36, generate targeted ES clones, and breed doubly-targeted mice:

As stated in the annual status reports for year one and two, early into this project, another group published a study in which they constitutively deleted a 4 Mb region of mouse chromosome 4 within the 1p36 syntenic region (Bagchi, et al, 2007), screened mouse embryonic fibroblast cells, and identified a candidate tumor suppressor in the region, CHD5. While this screen can be criticized for not being comprehensive in the genes screened and may not have included the region most frequently deleted in breast tumors, this was a significant result in the field. However, mice harboring this deletion have not developed breast tumors, which could suggest either the authors did not include the region most relevant to breast cancer, or that loss of this locus does not predispose cells in the mammary gland to transformation. Additionally, since initiating this project, we established that the HPRT-GFP construct we had proposed to use was not functional, necessitating the cloning of a vector with an alternative marker (the V5 epitope). Furthermore, while cloning alternative targeting vectors, the mouse genome assembly at the telomeric end of mouse chromosome 4 has changed twice, each time necessitating a total refinement of our targeting strategy and setting us back several months as we cloned new targeting construct plasmids (detailed in the status reports from the first two years). The current assembly does not map an available BAC clone to the region containing the most telomeric genes, making it difficult to

clone a targeting vector that would allow us to delete all of the genes in the 1p36 locus. While we have ongoing efforts to clone a large genomic sequence using long-range PCR on genomic DNA as a template, we have not been successful to date. Additionally, in summer 2008 my thesis committee concluded that, given the long time horizon for this project and the numerous technical setbacks experienced to date, I needed to focus on other aspects of my thesis project, most notably the Sleeping Beauty insertional mutagenesis approaches outlined in Task 3.

Task 2: Characterization of 1p36 deletion in the context of Her2-initiated oncogenesis:
As stated last year, since initiation of Task 2 is dependent on completion of Task 1, it has not yet been initiated. However, we have initiated efforts to combine the Sleeping Beauty system (Task 3) with MMTV-HER2/neu and MMTV-c-myc induced mammary tumors, described below.

Task 3: Identification of candidate genes using insertional mutagenesis (months 15-36)

As described in previous reports, the Sleeping Beauty insertional mutagenesis system works via a two part mechanism: 1) a transposon (T2/Onc, T2/Onc2, or T2/Onc3), a DNA sequence capable of activating or deactivating surrounding host genes, and 2) a transposase protein (SB10 or SB11) which excises the T2/Onc transposon and re-inserts it into a random location in the host genome. Cells harboring insertions conferring tumorigenic characteristics are selected and clonally expanded, allowing for identification of the insertion site and nearby genes.

We initially used the first-generation SB insertional mutagenesis system, consisting of a (presumably) constitutively expressed SB10 transposase under the control of a CAGGS (beta-actin) promoter. Transgenic mice harboring this construct and a transposon transgene did not succumb to tumors without a predisposing tumor suppressor knockout (Collier, et al, 2005). We combined this system with mice carrying the MMTV-HER2 transgene, susceptible to mammary tumors after a long latency. While we were able to successfully map transposon insertions, we did not observe any significant tumor acceleration compared to mice carrying MMTV-HER2 alone (described in the Annual Report from April 2008), nor did we see any transposase expression in tumors (described in the Annual Report from April 2007). The Largaespada group subsequently discovered that the CAGGS-SB10 construct was only expressed in muscle tissue, most likely due to a positional effect at the transgene insertion site. Consistent with this, most of the insertion sites we were able to map in tumors were also present in spleens from the corresponding mice, suggesting the transposase was active early in development but dormant during tumor development.

We next attempted to increase transposase activity using a Cre-activated Rosa-SB11 knock-in in combination with a high-copy transposon donor locus (T2/Onc2). We attempted to activate this construct with an MMTV-Cre transgenic mouse. However, consistent with other groups, we observed a high rate of leukemia formation in these mice, suggesting that the MMTV-Cre construct was active in the hematopoietic compartment, where the SB system was much more effective at inducing tumors. Among the mice that did not succumb to leukemia, none developed breast cancer, in spite of the MMTV-HER2 transgene they carried. We speculate this resistance is derived from the mouse strain background; unlike the first generation SB system, which was on an FVB/N background susceptible to MMTV-HER2 induced tumors, the second and third (described below) generations are on a mixed, mostly B6, genetic background that shows less susceptibility to these tumors. In parallel, we combined

this system with the MMTV-c-myc model of mammary tumorigenesis. Mice in this model were even more prone to leukemia; while two mice in our screen developed solid tumors which were unlikely to have originated in the mammary gland, most mice carrying the constructs succumbed to leukemia. The high rate of leukemia in these mice prevented us from generating any reasonable collection of mammary tumors for insertion site analysis.

We next crossed MMTV-HER2 mice to mice carrying a constitutive RosaSB11 knock-in construct, in combination with low copy transposons (T2/Onc and T2/Onc3, which contains a different promoter). We hoped leukemia in these mice would be modest (Collier, et al, in review) and would allow for acceleration of mammary tumors. Unfortunately, we have only observed four tumors to date in mice carrying both transposon and transposase constructs and the MMTV-HER2 transgene, a rate too low to effectively identify common insertion sites. Additionally, we observed two tumors in mice carrying only one transposon/transposase component (i.e., negative controls), suggesting tumor acceleration may not be greatly enhanced by the transposon system. This particular conformation was also complicated by the genetic background issue described above.

In an attempt to overcome strain issues and leukemia incidence, we next turned to an in vitro/transplant based strategy. Briefly, in this system, mammary epithelial cells (MECs) are isolated from donor mice, in this case, mice carrying a dormant transposon concatemer (T2/Onc, T2/Onc2, or T2/Onc3). These cells are cultured in vitro, and the SB transposase is then introduced with a viral vector. Virally-transduced cells are then transplanted into recipient mice in which the developing endogenous mammary epithelium has been surgically ablated. The transduced/transplanted MECs then repopulate the mammary fat gland, forming a normal branched ductal system, such that the mammary epithelium is genetically modified, but the surrounding tissue is wild-type.

We first tried to implement this system using MSCV-based retroviruses. As described in last year's report, cells isolated from MMTV-HER2;T2/Onc mice were transduced with a pMSCV-SB11-IRES-GFP (pMIG-SB11) and transplanted. Since these cells presumably had a growth advantage over normal cells (by virtue of the MMTV-HER2 transgene), endogenous epithelium was not ablated. While transplanted cells were viable (pictured in last year's report), we did not see the formation of fluorescent ducts, nor did we see clear tumor formation in the transplanted mammary glands.

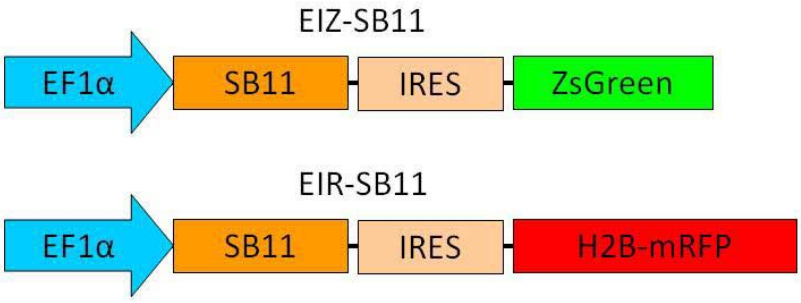

Figure 1: Lentiviral SB constructs.

We next upgraded to a lentiviral-based system. Lentiviruses can infect non-dividing cells and thus can infect the mammary stem cell subpopulation in a MEC culture, providing more efficient outgrowth of transduced cells. Importantly, in this system (unlike the retroviral system), transduced cells do not have to have a growth advantage to repopulate the mammary fat pad (Welm, et al 2008).

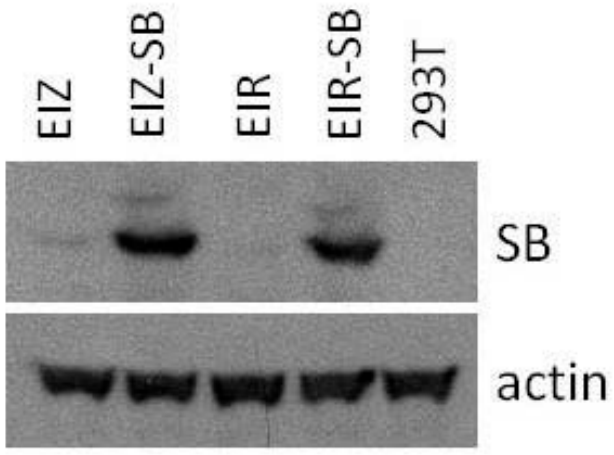

Figure 2: Expression of SB in 293T cells transduced with lentiviral constructs

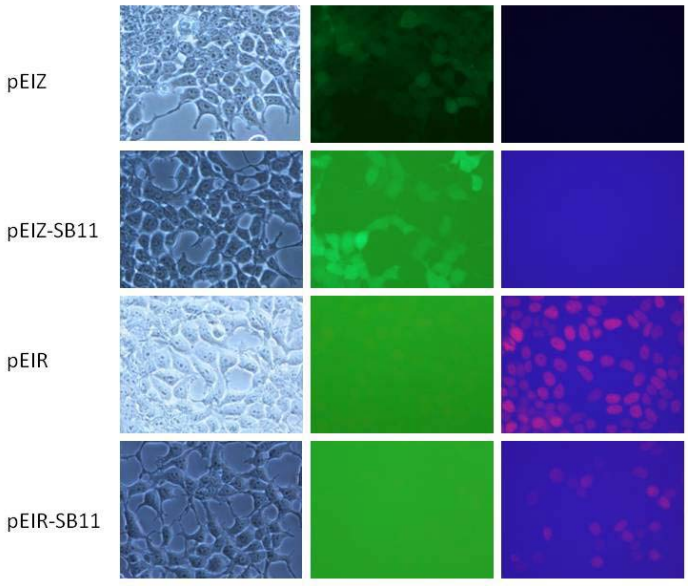

Figure 3: Expression of fluorescent markers in lentivirally-transduced 293T cells.

We have cloned the SB11 transposase into two lentiviral constructs, pEIZ and pEIR (Welm, et al 2008) to make the constructs pEIZ-SB11 and pEIR-SB11 (Figure 1). T hese c onstruct express t he t ransposase (Figure 2) and fluorescent m arkers (Figure 3) in t ransduced 293T cells.

We then isolated MECs from mice carrying the high-copy T2/Onc2 transposon, as well as mice carrying the transposon on a $p53^{+/-}$ background. MECs w ere t ransduced w ith either cont rol virus or SB11 virus at an MOI of 40. 300,000 cells were transplanted into cleared fat pads of 40 nude mice (20 receiving $p53^{+/+}$ cells and 20 receiving $p53^{+/-}$ cells) w ith the r ight g land receiving S B11-transduced cells and the l eft gland receiving ve ctor-transduced c ells. F orty mice ha ve be en unde r o bservation f or t umor formation f or 6 m onths. A c ontrol m ouse was di ssected t o establish that M ECs w ere reforming br anched epithelial s tructures; verification of the vi ability of th ese cells in comparison with wild-type glands a nd cleared fat pads is shown in Figure 4.

Should t his s ystem pr ove t o be e ffective a t inducing tum ors, it w ill provide a pow erful tool for f urther di ssection of br east-cancer pathways, as cells f rom an y t ransgenic or knockout donor mouse can be transduced and transplanted, a nd on e 10-week-old donor mouse can generate enough cells to transplant into 10 recipient mice in a pr ocess that takes less than a week. We hope that the cohort of mice c urrently under obs ervation w ill generate tumors at some point within the next 8-12 months, at which point we can perform insertion site analysis and identify genes altered in multiple tumors and provide proof-of-principle that transposon-based insertional mutagenesis is possible in the mouse mammary gland.

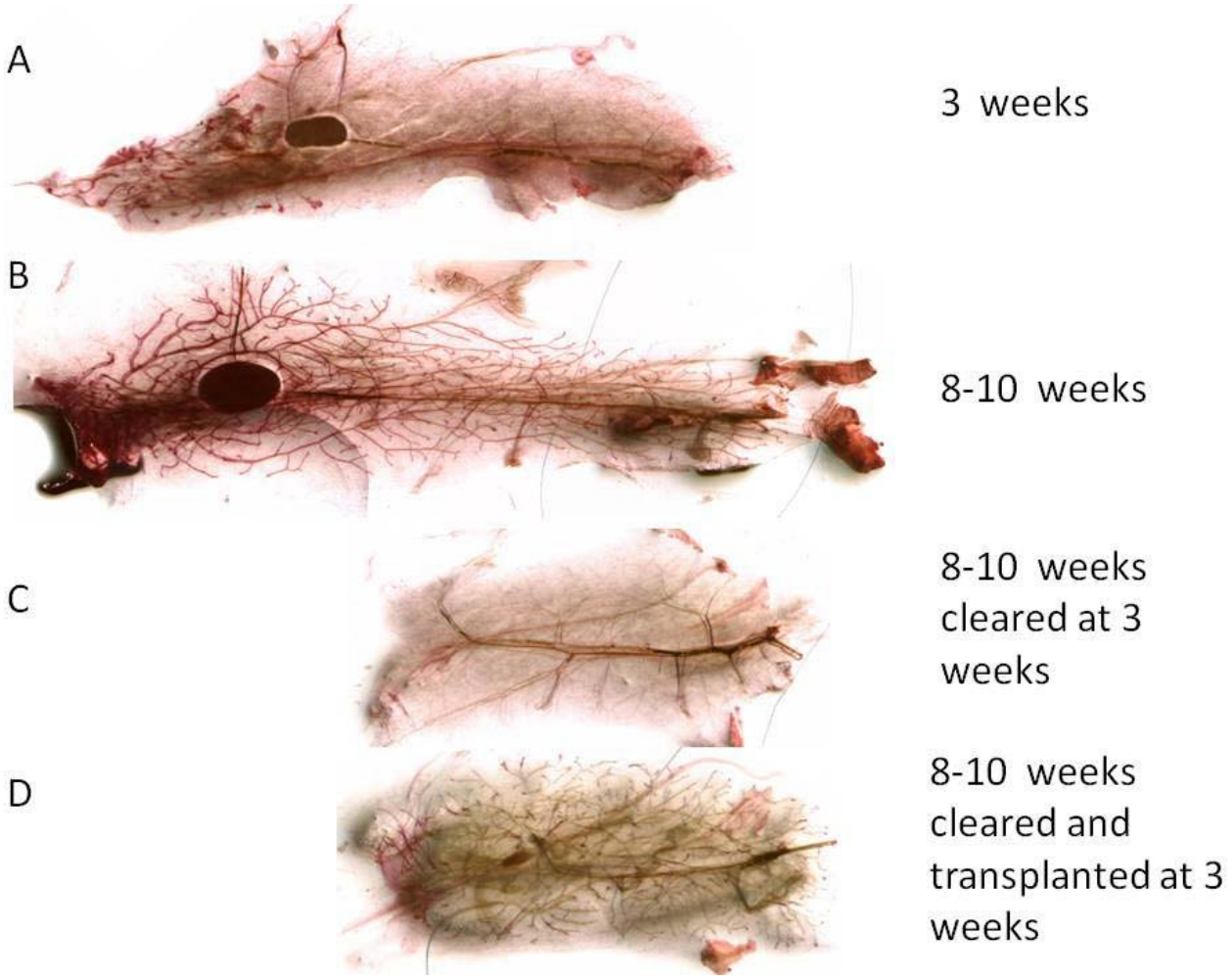

Figure 4: Reconstitution of mammary epithelium with transplanted cells. **A:** Mammary gland from a 3-week old mouse. Mammary epithelial cells (red Carmine stain) grow from the left, and do not pass the lymph node (center-left red oval) by 3 weeks of age. **B:** By 8 weeks of age, branched mammary epithelial structures have grown throughout the entire gland. **C:** In glands in which the portion of the gland including the lymph node and everything to the left have been surgically removed, no mammary epithelium is present in the 8-week old gland. **D:** In glands in which the endogenous mammary epithelium has been removed as in (C), and mammary epithelial cells from a donor mouse (transduced in vitro) have been transplanted on the right edge, the transplanted cells grow out to form a normal-looking branched epithelial structure.

KEY RESEARCH ACCOMPLISHMENTS:

- Generation of pEIZ-SB11 and pEIR-SB11constructs.
- Successful clearing and reconstitution of mouse mammary glands.
- Generation of 20 T2/Onc2 pEIR-SB11 mice and 20 T2/Onc2;$p53^{+/-}$ mice

REPORTABLE OUTCOMES:

Publications since award initiation:

1. Collier LS, Adams DJ, Hackett CS, Bendzick LE, Akagi K, Davies MN, Diers MD, Rodriguez FJ, Bender A, Tieu C, Matise I, Dupuy AJ, Copeland NG, Jenkins NA, J. Hodgson G, Weiss WA, Jenkins RB, Largaespada DA. **Whole-body Sleeping Beauty transponson mutagenesis can cause highly penetrant leukemia/lymphoma and rare high-grade glioma without associated embryonic lethality or genomic instability** (submitted)
2. Hackett C S, Geurts A M, Hackett PB. **Predicting preferential DNA vector insertion sites: implications for functional genomics and gene therapy.** Genome Biology 2007;8 Suppl 1:S12.
3. Cheng AJ, Ching Cheng N, Ford J, Smith J, Murray JE, Flemming C, Lastowska M, Jackson MS, Hackett C S, Weiss W A, Marshall GM, Kees UR, Norris M D, Haber M. **Cell lines from MYCN transgenic murine tumours reflect the molecular and biological characteristics of human neuroblastoma.** Eur J Cancer. 2007 Apr 19
4. Geurts AM, Hackett CS, Bell JB, Bergemann TL, Collier LS, Carlson CM, Largaespada DA, Hackett PB. **Structure-based prediction of insertion-site preferences of transposons into chromosomes.** Nucleic Acids Res. 2006 May 22;34(9):2803-11.

Abstracts:

1. Strain-specific penetrance and chromosome copy number variations in a mouse model for neuroblastoma. Christopher S. Hackett, J. Graeme Hodgson, Jian-Hua Mao, Denise Lind, Natalie Blades, Gary Churchill, Javed Khan, Pui-Yan Kwok, Allan Balmain, and William A. Weiss. AACR Mouse Models of Cancer Conference October 25-28, 2006.
2. **Transposon-based somatic mutagenesis for cancer gene discovery.** Lara S. Collier, David J. Adams, Laura E. Green, Eric P. Rahrmann, Michael N. Davies, Miechaleen D. Diers, Anthony J. Cox, Christopher S. Hackett, J. Graeme Hodgson, Adam J. Dupuy, Neal G. Copeland, Nancy A. Jenkins, William A. Weiss, Allan Bradley, Paul C. Marker and David A. Largaespada. AACR Mouse Models of Cancer Conference October 25-28, 2006.
3. **Structure-Based Prediction of Insertion-Site Preferences into Chromosomes of Vectors used for Gene Therapy.** P.B. Hackett, C.S. Hackett, and A.M. Geurts American Society for Gene Therapy, 2007.
4. **Sleeping Beauty insertional mutagenesis to identify genetic events in breast cancer progression**. Christopher S Hackett, Lara S. Collier, Adam J. Dupuy, David A. Largaespada, and William A. Weiss Beckman Center for Transposon Research, Minneapolis, MN, June 2007
5. **Sleeping Beauty insertional mutagenesis to identify genetic events in breast cancer progression.** Christopher S Hackett, Lara S. Collier, Adam J. Dupuy, David A. Largaespada, and William A. Weiss. AACR Advances in Breast Cancer Research, San Diego, CA, October 2007.
6. **Sleeping Beauty insertional mutagenesis and genetic linkage mapping identify loci governing tumor susceptibility in a mouse model for neuroblastoma.** Christopher S. Hackett, Timothy K. Starr, Lara S. Collier, Gary Churchill, Fernando Pardo-Manuel de Villena, Javed Khan, Pui-Yan Kwok, David Largaespada, Allan Balmain, and William A. Weiss. Advances in Neuroblastoma Research, Chiba, Japan, May 2008.
7. **Chromosome Engineering and Sleeping Beauty insertional mutagenesis to identify 1p tumor suppressors and other genetic events in breast cancer progression.** Christopher S Hackett, Timothy K. Starr, Lara S. Collier, Adam J. Dupuy, Nigel Killeen, Zena Werb, David A. Largaespada, and William A. Weiss. DOD Era of Hope Meeting, Baltimore, MD June 2008.

Awards:
AACR Scholar-in-Training Travel Award (AstraZeneca), AACR Mouse Models of Cancer Conference October 25-28, 2006.

CONCLUSION:

As of the previous annual report, we have de-emphasized generating a mosue knockout of the 1p36 region, in light of numerous unforeseen technical difficulties and the failure of another group to see breast tumors in a similar system. As described last year, we have also acquired a wide range of tools to express the SB11 transposase in breast tissue; two additional mouse constructs, and 3 novel retroviral viral constructs. As of last year, we had generated as significant cohort of mice and have isolated over 40 tumors. We sequenced insertion sites from 11 of these tumors, and have seen indications of common insertion sites. However, most of these insertion sites were also seen in normal spleen tissue, suggesting these insertions did not arise in the mammary gland and did not drive tumorigenesis. Additionally, tumors did not express transposase, and overall tumor onset was not significantly accelerated in transposon-bearing mice. We then tried next-generation Sleeping Beauty expression mice. However, these mice either did not express transposase at high enough levels to accelerate mammary tumors, or expressed transposase in the hematopoietic compartment and succumbed to leukemia (or both).

We have thus adopted a lentivirus-based system in which we isolate mammary epithelial cells carrying the transposon, transduce the SB transposase with a lentivirus, and transplant the cells into donor mice. This system eliminates the possibility of expression in the hematopoietic compartment while allowing high levels of transposase expression in mammary epithelial cells. Since this strategy has been only recently characterized and is technically more difficult than breeding multiple genetically engineered mice, we have established the ability to reconstitute mammary glands with transduced and transplanted mammary epithelial cells. We are currently observing the transplant recipient mice for tumor formation, which will provide proof of principle in the lentivirally-activated transposon-based insertional mutagenesis strategy in the mammary gland. This approach should provide a valuable tool with which to dissect various pathways in breast cancer.

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Bagchi A, Papazoglu C, Wu Y, Capurso D, Brodt M, Francis D, Bredel M, Vogel H, Mills AA. CHD5 is a tumor suppressor at human 1p36. Cell. 2007 Feb 9;128(3):459-75.

Collier LS, Carlson CM, Ravimohan S, Dupuy AJ, Largaespada DA. Cancer gene discovery in solid tumours using transposon-based somatic mutagenesis in the mouse. Nature. 2005 Jul 14;436(7048):272-6.

Welm BE, Dijkgraaf GJ, Bledau AS, Welm AL, Werb Z. Lentiviral transduction of mammary stem cells for analysis of gene function during development and cancer. Cell Stem Cell. 2008 Jan 10;2(1):90-102.

APPENDICES:

None.

SUPPORTING DATA:

Embedded in text.